ISSN (print) 0093-4666 © 2014. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/129.317

Volume 129(2), pp. 317–327 *October–December 2014*

New record of *Melanoleuca cinereifolia* in Himalayan moist temperate forests of Pakistan

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Abstract — Basidiomata of *Melanoleuca cinereifolia* have been collected underneath *Pinus wallichiana* trees in Himalayan moist temperate forests of Pakistan. Its identification was confirmed by nrDNA ITS sequence analysis. A detailed morphological description and illustrations are provided. This is the first report of *M. cinereifolia* from Asia.

Key words —Khyber Pakhtoonkhaw, pine forest, saprobic fungi, *Tricholomataceae*

Introduction

The Himalayan moist temperate forests of Pakistan, located at elevations of 1373–3050 m, are characterized by vigorous thick vegetation of conifers and some deciduous trees. These mixed forests have an average rainfall of 59.3 cm with humidity up to 57% (Champion et al. 1968). Although the Himalayas are considered one of twenty-five biodiversity hotspots, most mushroom species are yet to be reported (Myers et al. 2000; Razaq et al. 2013). During research on saprobic fungi from pine-dominated forests of Western Himalayas, *Melanoleuca cinereifolia* was identified as a new record for Pakistan. *Melanoleuca* Pat. is a cosmopolitan genus characterized by collybioid to tricholomatoid basidiomata, convex to slightly depressed pilei (often with a shallow umbo), emarginate to adnate to shortly decurrent lamellae, absence of a veil, white to pale-yellowish spore print, cutis to trichoderm pileipellis, hyaline spores with amyloid ornamentations, and absence of clamp connections (Singer 1986, Boekhout 1988, Vizzini et al. 2012).

Singer (1935, 1943, 1986), Kühner (1978), Bon (1978), and Boekhout (1988) gave much importance to morphology (e.g., pileus, stipe and lamellar colour, and stipe ornamentation) and anatomy (cystidial shape and the basidiospore Q value) for infrageneric classification in *Melanoleuca*. Vizzini et al. (2012) used ITS sequence analyses to check the traditional tripartite *Melanoleuca* subgeneric classification (*M.* subg. *Acystis* without cystidia; *M.* subg. *Urticocystis* with

urticiform cystidia; *M.* subg. *Melanoleuca* with macrocystidia). He concluded that *Melanoleuca* is a monophyletic genus in which only two emended subgenera, *Urticocystis* and *Melanoleuca*, are supported; species previously placed in *M.* subg. *Acystis,* shown to be polyphyletic and no longer accepted, are now supported in *M.* subg. *Urticocystis*.

Two other *Melanoleuca* species — *M. exscissa* (Fr.) Singer and *M. angelesiana* A.H. Sm. — have previously been reported from Pakistan (Ahmad 1980, Razaq 2013).

Materials & methods

Collection and morphological examination

The basidiome (MSM#005) was collected, photographed, vouchered, dried under fan heater, and characterized morphologically. Specimen sections were mounted in 5% KOH for examination under a MX4300H biological microscope (Meiji Techno Co., Ltd., Japan); phloxine was used to increase contrast, and Melzer's reagent was used to test for amyloidity of the basidiospores.

Measurements of anatomical features (basidiospores, basidia, cystidia, pileus hyphae and stipe hyphae) were calculated from at least 20 measurements made with an ocular micrometer and 100× oil-immersion objective; abbreviations include x = mean spore length and width for all spores measured, Q = spore length / width ratio. Line drawings were made with a Lucida camera. Color designations are from Munsell (1975).

DNA extraction, PCR amplification, DNA sequencing

Genomic DNA was extracted from a small piece of pileus by a modified CTAB method (Bruns 1995). The internal transcribed spacers (ITS1, ITS2) + 5.8S region of the nuclear ribosomal RNA gene were targeted by the ITS1F/ITS4 primer pair (White et al. 1990; Gardes & Bruns 1993) using an Extract-N-Amp plant DNA extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). The PCR amplification parameters were: initial denaturation (94 °C for 1 min), 35 cycles (94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min), and final extension 72 °C (8 min). Amplified PCR products were sent for purification and bidirectional sequencing to Macrogen (Korea).

Sequence alignment and phylogenetic analysis

The nrITS sequence from MSM#005 comprised 740 base pairs. Initial blast analysis of nucleotide sequences revealed that the specimen showed 99% maximum identity with *Melanoleuca* aff. *cinereifolia*, *M. cinereifolia*, *M. communis* (GenBank JX429210, JX429108, JX429197, JX429207). GenBank sequences were selected based on the phylogenetic studies on *Melanoleuca* by Vizzini et al. (2012) and Sánchez-García et al. (2013).

Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Following Dentinger et al. (2011) for complete ITS sequences, all sequences were trimmed with the conserved motifs 5′-(…GAT)CATTA– and –GACCT(CAAA…)-3′ and the alignment portion between them were included in the analysis. Gaps were treated as data for construction of phylogeny.

The sequence from MSM#005 was aligned with GenBank sequences of *M. cinereifolia* and other related taxa (Table 1) by Muscle using the default setting in Molecular

Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2011). A phylogenetic tree was constructed with the Maximum Likelihood (ML) algorithm and Neighbor-Joining method, Jukes & Cantor (1969) model of nrITS sequences, and nearest-neighbor-interchange (NNI) as ML heuristic search method using MEGA5 software (Tamura et al. 2011). Phylogeny was tested by a bootstrap value of 1000 replicates, and bootstrap values >50% are cited in the tree.

Sequence of *M. cinereifolia* generated for this study was submitted to the Genbank and the accession numbers for this, as well as for other closely related taxa used in the phylogenetic analysis are cited in phylogenetic tree. Percent Identities (PID) and DNA divergence were calculated by DNAStar.

Table 1. *Limnoperdon* and *Melanoleuca* ITS-rDNA sequences used in the phylogenetic analysis. New sequence indicated in bold.

Taxon	Collection/Voucher	Country	Genbank #
L. incarnatum	IF030398	Japan	DQ097363
M. angelesiana	ANC M0203	Italy	JN616420
	ANC M0203	Italy	JN616421
M. brevipes	4574	Italy	JF908352
M. aff. *cinereifolia*	FCME11225	Mexico	JX429108
	IBUGJS89	Mexico	JX429210
M. cinereifolia	319	Italy	JN052137
	1471	Italy	JN052138
	MSM#005	**Pakistan**	**KJ182965**
M. cognata	13939	Italy	JF908360
	ANC M0170	Italy	JN616425
M. communis	XAL Murrieta1025	Mexico	JX429204
	FCME 17116	Mexico	JX429207
	ENCB Guzman 6329	Mexico	JX429226
M. decembris	ANC M0197	Italy	JN616426
	ANC M0200	Italy	JN616428
M. exscissa	ANC M0210	Italy	JN616433
	ANC M0213	Italy	JN616436
M. grammopodia	ANC M0217	Italy	JN616439
	ANC M0218	Italy	JN616440
M. heterocystidiosa	ANC M0174	Italy	JN616444
	ANC M0175	Italy	JN616445
M. nivea	MCVE 9578	Italy	JN392452
	ANC M0177	Italy	JN616450
M. paedida	ANC M0189	Italy	JN616452
	ANC M0190	Italy	JN616453
M. pseudoluscina	ANC M0194	Italy	JN616458
	ANC M0195	Italy	JN616459
M. strictipes	ANC M0172	Italy	JN616465
	ANC M0173	Italy	JN616466
M. subpulverulenta	ANC M0004	Italy	JN616472
	ANC M0178	Italy	JN616473
M. substrictipes	ANC M0214	Italy	JN616474

Fig. 1. *Melanoleuca cinereifolia* (MSM#005). Basidiomes. Scale bars: 10 mm.

Taxonomy

Melanoleuca cinereifolia (Bon) Bon, Docum. Mycol. 9(33): 71 (1978). Figs 1–3

Description based on a single basidiome:

Pileus 21 mm diam., plano-convex, flat, thin (collybioid); margin straight or flaring, smooth; surface dull, off-white to grayish near margin (5R9/2), central disc chocolate brown (5YR1/2). Lamellae free or approximate, close, off-white to gray (5YR9/2). Stipe 26 mm, central, subequal, often with subbulbous base, hollow, fibrillose, light brown (5YR7/4). Rhizomorphs white, few. Odor and Taste not recorded. Basidiospores 4.3–6.1 × 7.2–9.5 µm [x = 5.4 × 8.5 µm, Q = 1.57], oblong or ellipsoid, apiculus absent, warty, thin-walled, hyaline in KOH, amyloid. Basidia 8.5–11.8(–13) × 28.7–38.2(–44.8) µm, clavate, mono-, bi-, or tetra-spored, thin-walled, hyaline in KOH; sterigmata 1.8–4.2(–5.1) µm. Trama hyphal, 6.1–8.9 µm, thin-walled, hyaline in KOH. Cheilocystidia 11.8–14(–16.9) × 47–71(–77.4) µm, lageniform with crystals at the apex, sometimes septate, hyaline, slightly thick walled, up to 1 µm. Pleurocystidia similar to cheilocystidia. Pileipellis a cutis, hyphae cylindrical, 9–16 µm, thin-walled, hyaline in KOH. Stipe Hyphae cylindrical, 5–11.8(–14.8) µm, hyaline to pale yellow in KOH. Clamp connections absent.

Material examined: **PAKISTAN**, **Khyber Pakhtoonkhaw**, Shangla, Yakh Tangay, under *Pinus wallichiana* A.B. Jacks., 2 September 2013, coll. M. Saba & A.N. Khalid, MSM#005 (LAH; GenBank KJ182965).

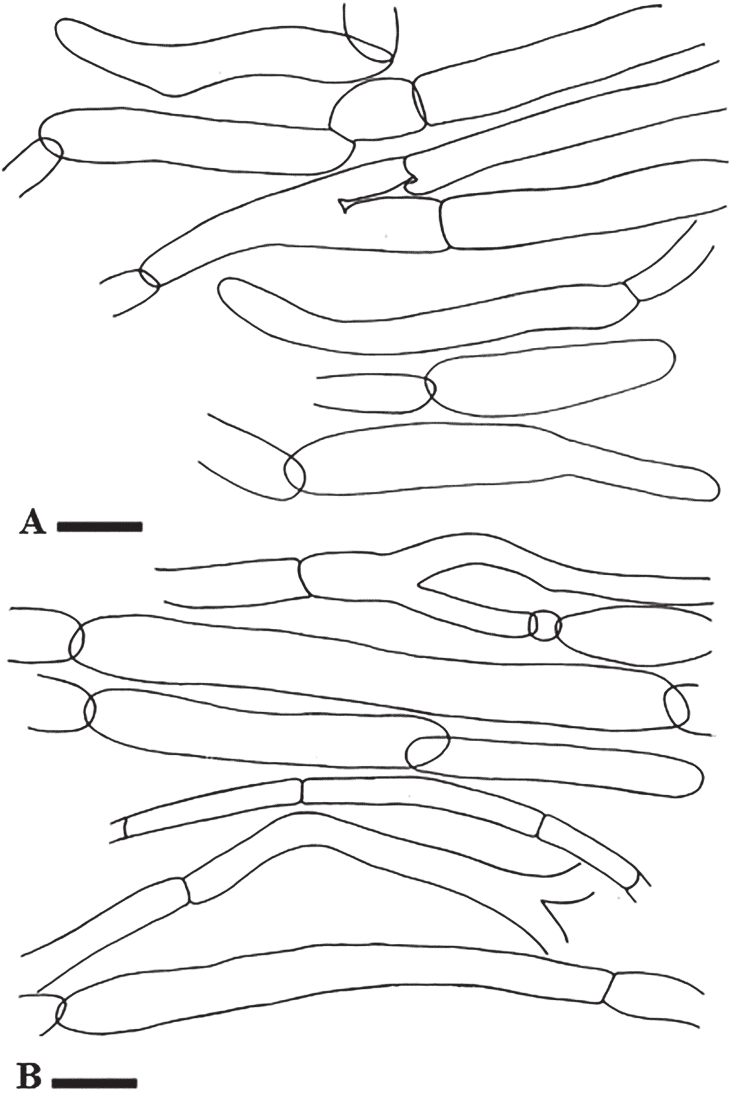

Fig. 2. *Melanoleuca cinereifolia* (MSM#005). A, pileipellis; B, stipitipellis. Scale bars: A = 20 µm, B = 16 µm.

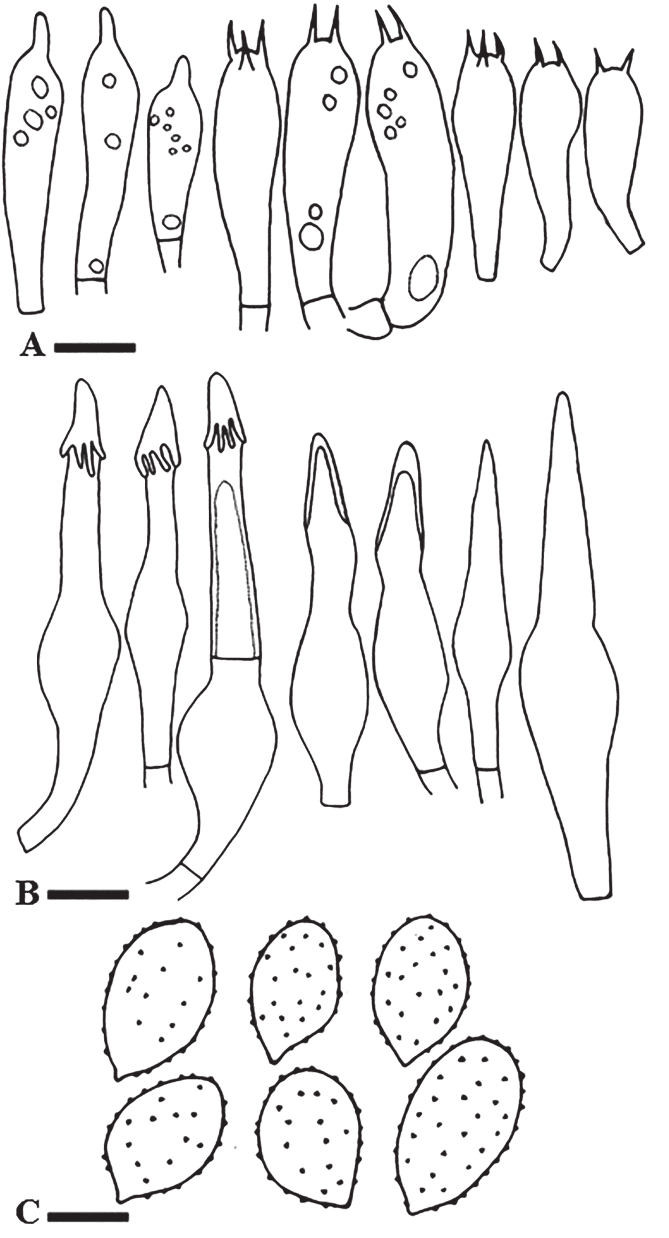

Fig. 3. *Melanoleuca cinereifolia* (MSM#005). A, basidia; B, cheilocystidia; C, basidiospores. Scale bars: A, B = 10 µm; C = 3.6 µm.

Results

The phylogenetic trees were constructed with the Maximum Likelihood algorithm (Fig. 4) and Neighbour-Joining method (Fig. 5), using Jukes & Cantor (1969) model of nrITS sequences and nearest-neighbor-interchange (NNI) as ML heuristic search method using MEGA5 software (Tamura et al. 2011). Codon positions included were 1st+2nd+3rd+Noncoding. The

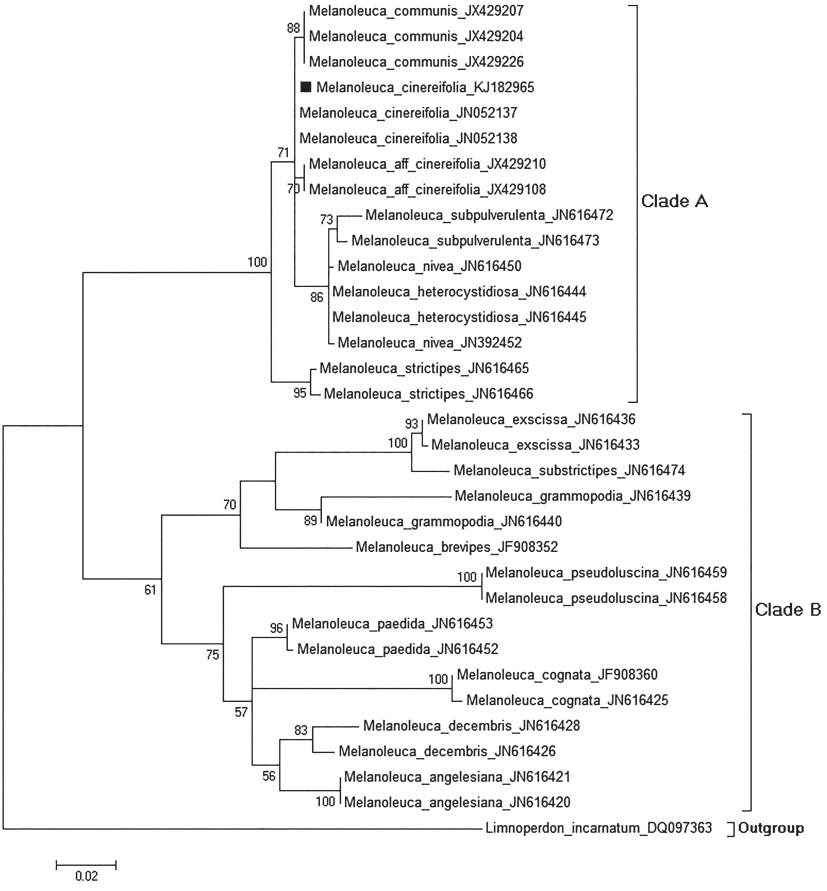

Fig. 4. Phylogenetic relationship of *Melanoleuca cinereifolia* with other *Melanoleuca* spp. based on Maximum Likelihood method inferred from nrITS sequences. The tree with the highest log likelihood (–3381.2856) is shown. Bootstrap values >50 (1000 replicates) are shown below the branches, and the percentage of trees in which the associated taxa cluster together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

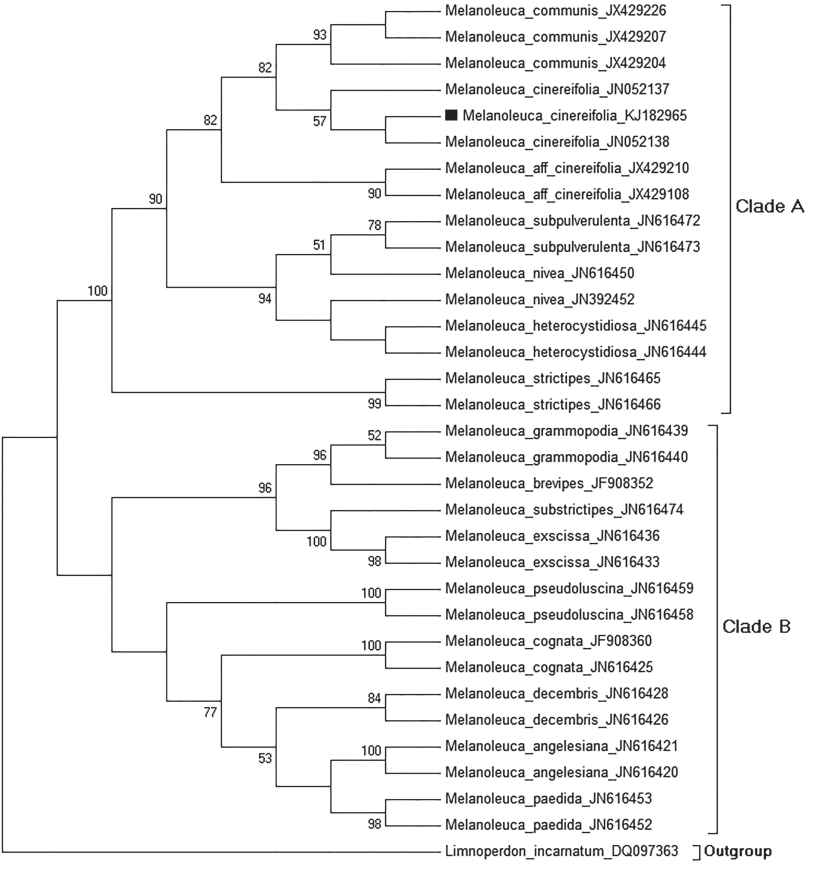

Fig. 5. Phylogenetic relationship of *Melanoleuca cinereifolia* with other *Melanoleuca* spp. based on Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site.

final dataset contains 33 nucleotide sequences. *Limnoperdon incarnatum* (DQ097363) was used as outgroup based on results from Vizzini et al. (2012).

After removing and editing the ambiguous letters from aligned datasheet, a total of 711 characters were subjected for phylogenetic analysis, of which 435 characters were conserved, 274 were variable, 186 were parsimony informative, and 87 were singletons.

The percentage similarity was calculated by MegAlign (DNA Star Inc.). *Melanoleuca cinereifolia* showed 99.4% identity and 0% genetic divergence with sequences retrieved from GenBank (JN052137, JN052138), 99.4% identity and 0.5% divergence with *M.* aff. *cinereifolia* sequences (JX429108, JX429210), and 99.3% identity and 0.5% divergence with *M. communis* (JX429226, JX429204, JX429207).

Discussion

Melanoleuca cinereifolia and *M. communis* M. Sánchez-García & J. Cifuentes closely resemble each other. Both species show 99% maximum similarity to the present species in the Blast search and probably represent the same taxon. In the phylogeny MSM#005 clusters with *M. cinereifolia* and morphologically, it differs from *M. communis* in basidiome and pileus size and pileus and lamellar colour. *Melanoleuca cinereifolia* is usually characterized as a basidiome with a short stipe, grey lamellae, lageniform cystidia that grows in sand dunes (Vizzini et al. 2012). Lantieri et al. (2009), describe it from Italy as frequent on sand dunes characterized by *Cypero capitati-Agropyretum juncei* and as well as at higher elevations characterized by *Medicagini marinae-Ammophiletum australis*. In Pakistan, our *M. cinereifolia* specimen was collected growing on forest floor rich in organic matter in a pure *Pinus wallichiana* forest. Sánchez-García et al. (2013) collected *M.* cf. *cinereifolia* in mountainous mesophilic forests in Mexico.

Other *Melanoleuca* species known from Pakistan are *M. exscissa* and *M. angelesiana* (Ahmad 1980; Razaq 2013). Unlike *M. cinereifolia, M. exscissa* has a urticiform *exscissa* cystidial type while *M. angelesiana* lacks cystidia.

Identification of *Melanoleuca* species is difficult because most morphological characters are homoplastic and influenced by environmental factors (Vizzini et al. 2012), making molecular analysis necessary for species identification.

Our phylogenetic trees (Figs 4, 5) divide *Melanoleuca* into two major clades, supporting recognition of two subgenera (*Melanoleuca* and *Urticocystis*). Clade A in which the Pakistani specimen is nested is supported by a robust (100) bootstrap and includes taxa mainly with lageniform or fusiform macrocystidia. Clade B, which is supported by only 56 bootstrap, includes all taxa with urticoid cystidia, some species lacking cystidia, and the macrocystidiate *M. cognata* complex. *Melanoleuca exscissa* and *M. angelesiana* reported from Pakistan cluster in clade B. Our phylogeny agrees closely with the results reported by Vizzini et al. (2012). Moreover, percent identity and genetic divergence support the identity of MSM#005 as *M. cinereifolia*, as inferred from the phylogenetic tree.

Acknowledgments

We are highly indebted to Higher Education Commission (HEC), Pakistan, for funding this project under Phase II, Batch I, Indigenous PhD fellowships Program for 5000 scholars. We are cordially grateful to Dr. Alfredo Vizzini (Dipartimento di Scienze della Vita e Biologia dei Sistemi-Universitá degli Studi di Torino, Viale Mattioli, Torino, Italy), Dr. Omar Perdomo (Dominican Society of Mycology, Dominican Republic), and Dr. Vladimír Antonín (Moravian Museum, Czech Republic) for critically reviewing the manuscript and their valuable comments. We are thankful to all lab fellows for accompanying us on the field trips.

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